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# Evaluation of Maternal Serum α-Foetoprotein Assay Using Dry Blood Spot Samples

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**Summary:** The quantification of α-foetoprotein in dry blood spots from pregnant women was evaluated, using a conventional radioimmunoassay (RIA) with a monospecific antibody. The stability of α-foetoprotein in dry blood spots on filter paper was evaluated with respect to mailing, distances travelled, and the existence of high summer temperatures in our region. The results obtained show that the blood α-foetoprotein is stable on dry filter spots sent by mail and is stable for up to four weeks at 4, 25 and 37 °C. The analytical method used has a minimal detectable concentration of 10 ± 1.9 international kilo-units/l. Both inter- and intra-assay variabilities are smaller than 10% and this method can provide results comparable with those of conventional serum assays. Results from dry blood spots and serum samples (the latter analysed by both RIA and two-site enzyme immunoassay) exhibited a good correlation ($r = 0.98$ and $r = 0.97$, $p < 0.001$). The design of the assay and the nature of the samples make this method suitable for a screening programmes for the antenatal detection of open neural tube defects.

## Introduction

α-Foetoprotein is, besides albumin, the major protein in foetal circulation. It consists of a single polypeptide chain with 590 amino acids, 3.4–4% carbohydrate, and a molecular weight of about 69 000 (1). α-Foetoprotein testing for the detection of neural tube defects is based on the observation, reported more than a decade ago, that this protein is detectable in maternal serum and amniotic fluid, and that, in the presence of an open neural tube defect, its concentration markedly exceeds normal limits (2–4). Neural tube defects are among the most common birth defects, occurring with a frequency of one to two cases per 1000 live births, 95% of them with no prior family history of such disorders (5–8).

Testing of α-foetoprotein in maternal serum provides a means of screening the population for antenatal diagnosis of neural tube defects. The test allows the selection of women with high risk pregnancies. Thus, if the maternal serum α-foetoprotein value is above the norm for the population group to which the patient belongs, a protocol of diagnosis is initiated, including a second serum α-foetoprotein test, ultrasonography and amniotic fluid α-foetoprotein and acetylcholinesterase tests (5–12). The most common procedures used for maternal serum α-foetoprotein testing have been radioimmunoassays (RIA) on serum samples (13–16). The measurement of α-foetoprotein in dry blood spots, from newborn children and pregnant women, has been previously described for the adaptation of RIA and immunoradiometric kits, showing a good concordance between the dry blood and serum RIA tests (17–19).

In this paper, we report our experience with the development of a competitive single-antibody RIA as a inexpensive method for α-foetoprotein determination on dry blood spots. These samples were used in a screening programme in our region with locations up to 250 km apart. Its use eases the sending of samples by mail. However, in summer the temperatures are around 40 °C. This necessitates a study of α-foetoprotein stability in dry blood spots subjected to high temperatures. We report the results obtained, recovery and precision and the estimation of α-foetoprotein on a wide population of pregnant women, using dry blood spot samples.

## Materials and Methods

### Specimens

1016 dry blood specimens from pregnant women were used in this study. Blood samples, obtained by vein puncture, were applied as 1 cm diameter spots to filter paper (No. 903 Schleicher and Schuell), air dried at room temperature and routinely stored in plastic bags at 4 °C. For the assay, a disc, 6.5 mm in diameter, punched from between the center and edge of each spot was placed directly into a 12 × 75 mm polystyrene test tube. Serum specimens were obtained from 200 pregnant women and stored at −20 °C until their analysis. α-Foetoprotein concentrations were measured and the results were compared with those for dry blood spot samples obtained from the same individuals.

### Preparation of samples

All dry blood spot specimens were eluted with a 50 mmol/l phosphate-buffered saline solution (pH 7.5). The punched disc was eluted by allowing it to stand for 16 hours at 4 °C with 200 µl of the described phosphate buffered saline. Experiments with $^{125}$I-labelled human α-foetoprotein showed that the analytical recovery of α-foetoprotein from dry blood spots was better than 90%.

### Stability of α-foetoprotein in dry blood spot samples

To determine the α-foetoprotein stability in the dry state we spotted 10 blood samples from pregnant women on filter paper as described above. After the initial concentrations had been determined, the blood spots were stored at 4, 25 and 37 °C or were air-mailed in summer to three cities up to 250 km away. The mailed samples were returned by mail within one week and α-foetoprotein levels in these samples and in those which had been kept at 4, 25 and 37 °C, were determined.

### Dry blood spot α-foetoprotein RIA components

Monospecific rabbit anti-human α-foetoprotein, as well as purified human α-foetoprotein were purchased from Dako Co. (Santa Barbara, CA, USA). The α-foetoprotein was iodinated with $^{125}$I using the method of *Kirkpatrick & Nakamura* (13). The anti-human α-foetoprotein serum was titrated by incubating it in serial dilutions (1/1000 to 1/200 000) with 175 pg of $^{125}$I-labelled α-foetoprotein in 0.1 mol/l phosphate buffer, 1 g/l ovalbumin, pH 7.5. The immunoprecipitating reagent (protein-A-bearing Cowan I strain *Straphylococcus aureus*, 10 g/l) was supplied by CSIC (Madrid, Spain). Dry blood standards were prepared by diluting the WHO reference standard (12-388, Statens Serum Institute, Copenhagen, Denmark) with α-foetoprotein-free whole blood, to give a final range from 0 to 320 international kilo-units/l of serum. Dry blood specimens were then prepared as previously described and stored in plastic bags at 4 °C until further use. In the assay, the buffer used for dilution of the antibody and the $^{125}$I-labelled α-foetoprotein was 50 mmol/l phosphate buffered saline, 2.5 g/l ovalbumin, pH 7.5.

### Serum α-foetoprotein RIA components

These components are basically the same as those of the α-foetoprotein RIA dry blood spots. In this case, however, the reference standards were prepared in plasma instead of dry blood and were kept at −20 °C until their use (before 3 months). The buffer used for dilution of the serum sample, the antibody and the $^{125}$I-labelled α-foetoprotein was 0.1 mol/l phosphate buffer, 1 g/l ovalbumin, pH 7.5.

### Serum α-foetoprotein enzyme immunoassay components

Monospecific rabbit anti-human α-foetoprotein (Dako, Co., Santa Barbara, CA, USA) was labelled with horseradish peroxidase, using the periodate oxidation conjugation method (20). The same unlabelled antibody was immobilized on Dinatec microelisa M-129 microplates (Florida, USA). For this purpose, 100 µl of 1/100 antibody dilution (in phosphate buffered saline, 50 mmol/l, pH 7.2) was added to each well, followed by a 24 hour incubation period at 4 °C. The wells were then washed 3 times with 1 ml Tween 20 in 1 l of 50 mmol/l phosphate buffered saline solution, and 200 µl of 5 g/l human albumin in phosphate-buffered saline, 50 mmol/l, pH 7.2, were added. After 4 hours incubation at 25 °C, the content of the wells was discarded and the microplates were stored at −40 °C until use. The enzyme label was assayed by oxidation of an *o*-phenylenediamine solution composed of 0.1 mol/l citrate buffer, pH 5.1, containing 5 mmol/l $H_2O_2$ and 16 mmol/l *o*-phenylenediamine dihydrochloride (Sigma). The purified α-foetoprotein and serum α-foetoprotein standards were the same as those used in the RIA method.

### Performance of the assays

Dry blood spots and serum samples were used for the α-foetoprotein assay. Each spot was eluted into 200 µl of 2.5 g/l ovalbumin and 0.5 mmol/l thimerosal (Sigma Chemical Co., St. Louis, MO, USA) in 50 mmol/l phosphate-buffered saline, pH 7.5 (Buffer A), 100 µl of buffer A with 25 mmol/l EDTA (Merck, Darmstadt, GFR)) (Buffer B), and 100 µl of antiserum in a final dilution of 1/25 000. For serum α-foetoprotein RIA, 100 µl of 1/20 serum dilution were added to 100 µl of antibody dilution and 100 µl of 0.1 mol/l phosphate buffer, 1 g/l ovalbumin, pH 7.5. After overnight incubation at 4 °C, 100 µl of $^{125}$I-labelled α-foetoprotein (approx. 25 000 counts/min) were added and incubation continued for a further 72 and 48 hours for dry blood spot and serum samples respectively. To precipitate the immune complexes, 1 ml of a solution containing 65 ml immunoprecipitating reagent in 1 l of 50 mmol/l phosphate-buffered saline pH 7.5 was added to each tube, then centrifuged (4000 *g*, 20 min, 4 °C), supernatants discarded, and the radioactivity in the pellet measured in a gamma counter (Kontron Analytical, Münchenstein, Switzerland). Data evaluation was computer-aided, using a semi-logarithmic fit.

For the enzyme Immunoassay (EIA) determination, 100 µl of 50 mmol/l phosphate-buffered saline, 5 g/l bovine albumin, pH 7.2 and 10 µl of standard or serum samples were transfered to a well coated with an anti-α-foetoprotein antibody. The mixture was stirred and incubated for 1 hour at 37 °C, then the wells were washed 3 times with the Tween 20 in phosphate-buffered saline. Then, 100 µl of a 1/200 dilution of labelled antibody (in 50 mmol/l phosphate-buffered saline, 5 g/l bovine albumin, pH 7.2) were added to each well and the wells were incubated for 1 hour at 37 °C. The wells were washed 3 times again and finally 100 µl of *o*-phenylenediamine solution were added. After 30 min of incubation at 25 °C, the peroxidase activity was stopped with 100 µl of 1 mol/l $H_2SO_4$ and determined by measuring the resulting colour at 492 nm in a Titerket multiscand spectrometer (Flow Laboratories, Ayrshire, Scotland).

### Statistical methods

Results are expressed as mean ± standard error (SEM); standard deviation (SD) and coefficient of variation (CV) are also included. Statistical analyses were performed using one-way analyses of variance (ANOVA); significant differences between groups were determined by the Student Newman Reuls test.

## Results

### Assay sensitivity and precision

The standard RIA assay curve for dry blood spot samples extended from 0 to 320 international kilo-units/l (fig. 1). Using the formula given by *Rodbard* (21) or 2 × SD of blank measurement value, we determined the lower limit of sensitivity to be 10 ± 1.9 international kilo-units/l (means ± SEM, n = 10) or 10.5 ± 1.7 international kilo-units/l (means ± SEM, n = 10), respectively. Within-run CVs of 19.5, 7.0 and 8.2% were determined by testing 10 discs from each of three spots containing 10, 40 and 160 international kilo-units/l, respectively. The respective between-run CVs were 16, 7 and 10%.

### Recovery

Analytical recovery of WHO stock material was used to assess the calibration accuracy of our method. For this purpose, we added aliquots of WHO reference standard stock material to α-foetoprotein-free whole blood in amounts to cover the expected concentration range. The amounts found were compared with those calculated and the results are summarized in table 1.

Tab. 1. Recoveries of α-foetoprotein from dry blood spot samples.

| α-Foetoprotein (International kilo-units/l) | | |
|---|---|---|
| Expected | Found mean ± SD, n = 3 | Recovery (%) mean ± SD, n = 3 |
| 10 | 10.7 ± 2.6 | 107.4 ± 26.4 |
| 40 | 43.8 ± 2.5 | 109.4 ± 6.3 |
| 160 | 163.6 ± 8.7 | 102.3 ± 6.7 |

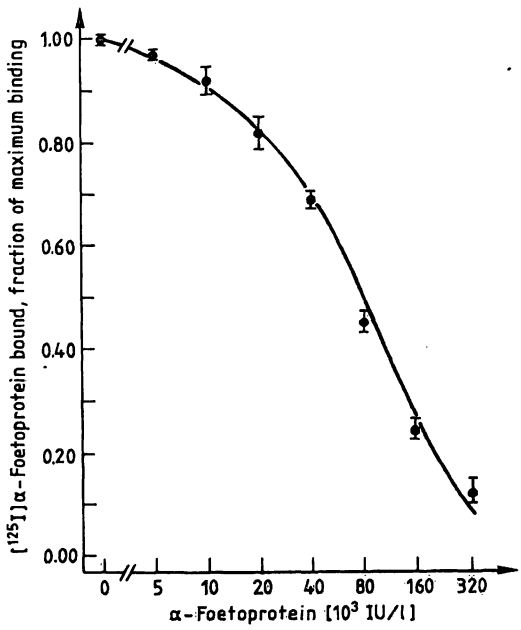


Fig. 1. RIA calibration curve for α-foetoprotein. Results shown are the mean ± SD of triplicates.

### Correlation

For our comparison study we used 200 dry blood and serum samples from the same individuals, who were pregnant women of 10 to 39 weeks gestation. α-Foetoprotein contents in serum, determined by both RIA and EIA methods, correlated significantly with those obtained in the dry blood spots, as shown in figures 2 and 3. The correlation coefficients obtained were 0.98 and 0.97 ($p < 0.001$), respectively.

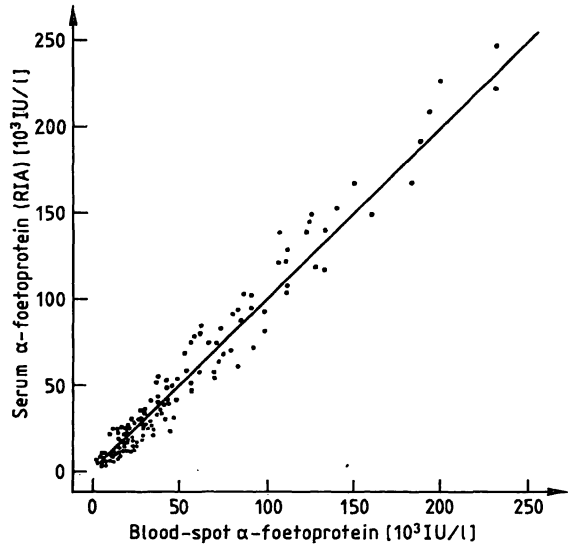


Fig. 2. Relationship between RIA serum and dry blood spot results.
n = 157, y = − 1.76 + 1.03x, r = 0.98, p < 0.001.

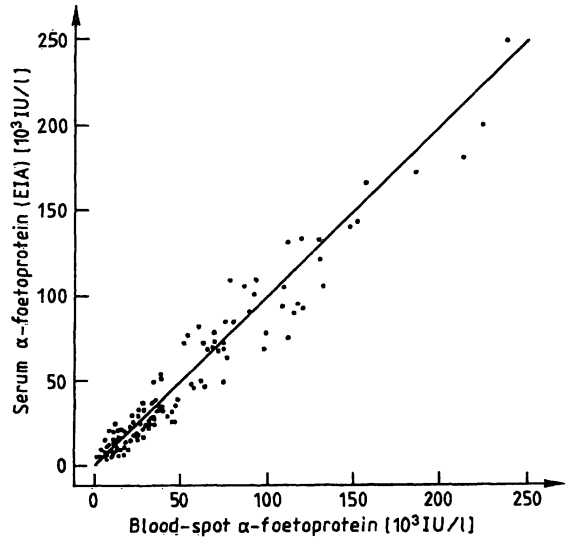


Fig. 3. Relationship between EIA serum and dry blood spot results.
n = 140, y = 1.56 − 0.92x, r = 0.97, p < 0.001.

## Stability of α-foetoprotein in blood spot samples

The effect of mailing was analysed on 10 samples which were obtained from pregnant women with gestational age ⩾ 16 weeks (tab. 2). The test for significant differences of two samples was applied to three groups, each including α-foetoprotein concentrations initially obtained and those measured in dry blood spots returned by mail from the three cities previously described. No significant differences were found. Table 2 shows both the initial concentrations and those determined in mailed samples. Thermal stability at 4, 25 and 37 °C was analysed on the above described samples. The results obtained were approximately the same (fig. 4) and we did not observe significant changes in the α-foetoprotein content between the initial concentrations and those of the different groups studied. The results obtained show that α-foetoprotein in dry blood spots remains stable during mailing and exposure to 4, 25 and 37 °C for at least four weeks.

## Effect of haematocrit

We assessed the effect of haematocrit by assaying dry blood specimens prepared from an initial blood sample, supplemented with α-foetoprotein to give approximately 20, 40, 80 and 160 international kilo-units/l α-foetoprotein (tab. 4). The initial haematocrit was 0.35 and we increased the haematocrit to 0.40 and 0.47 by removing part of the plasma. Haematocrits between 0.35 and 0.47 did not affect the results for α-foetoprotein in dry blood spot samples ($P < 0.05$, Anova, file = 3, sample size = 60).

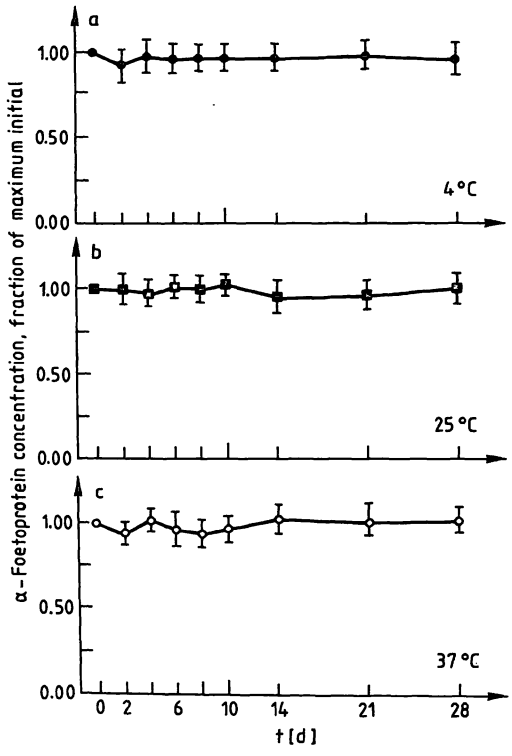


Fig. 4. Storage stability of α-foetoprotein at different temperatures.
Results shown are the mean ± SD, n = 10.
a) 4 °C
b) 25 °C
c) 37 °C

Tab. 2. Stability of α-foetoprotein in dry blood spots returned by mail from three cities (a, b, c) from 120 to 250 km from the laboratory.

| Gestational age (weeks) | α-Foetoprotein (International kilo-units/l) | | | |
|---|---|---|---|---|
| | Initial | Upon return by mail | | |
| | | a | b | c |
| 16 | 16 | 19 | 19 | 15 |
| 25 | 65 | 64 | 64 | 67 |
| 16 | 40 | 43 | 40 | 42 |
| 17 | 5 | 4 | 4 | 3 |
| 27 | 58 | 60 | 55 | 61 |
| 23 | 13 | 14 | 15 | 11 |
| 19 | 21 | 20 | 24 | 22 |
| 33 | 73 | 77 | 75 | 79 |
| 22 | 41 | 40 | 42 | 39 |
| 30 | 47 | 49 | 49 | 46 |

Tab. 3. Effects of haematocrit on α-foetoprotein concentration.

| α-Foetoprotein (International kilo-units/l) mean ± SD, n = 5 | | |
|---|---|---|
| Haematocrit 0.35 | Haematocrit 0.40 | Haematocrit 0.47 |
| 24.3 ± 2.7 | 21.1 ± 2.7 | 20.5 ± 0.6 |
| 35.9 ± 1.6 | 39.0 ± 1.9 | 38.6 ± 1.2 |
| 81.5 ± 1.8 | 78.0 ± 2.4 | 81.1 ± 1.5 |
| 149.2 ± 4.3 | 146.0 ± 5.5 | 152.6 ± 3.8 |

Tab. 4. Values obtained for α-foetoprotein in maternal dry blood spot samples for patients screened at 16 to 20 weeks of pregnancy. Pregnancies subsequently involving neural tube defects, and multiple pregnancies have not been included in this table.

| Gestational age | | Median | Mean ± SD |
|---|---|---|---|
| weeks | number | International kilo-units/l | |
| 15 | 37 | 18 | 22.4 ± 14.8 |
| 16 | 43 | 26 | 27.3 ± 14.3 |
| 17 | 43 | 23 | 26.3 ± 13.9 |
| 18 | 34 | 31 | 35.1 ± 18.6 |
| 19 | 43 | 38 | 43.9 ± 23.5 |
| 20 | 34 | 34 | 37.8 ± 18.8 |

## Clinical studies

We evaluated 1016 maternal blood spots from pregnant women for α-foetoprotein content. The pregnant women were from an unselected population, ranging from 5 to 42 weeks of gestation. The α-foetoprotein maternal dry blood spot distribution throughout pregnancy is shown in figure 5. Concentrations of α-foetoprotein are expressed as median and 2.5 × median with respect to each gestational age. Table 4 shows values for median and mean ± SD, for gestational weeks 15 to 20, obtained with the dry blood spot samples. These specimens were from non-neural tube defect singleton pregnancies seen at our hospital. The gestational age of each pregnancy was defined as the weeks of pregnancy completed from the first day of the last menstrual period. The best time for detecting open neural tube defects by measuring maternal serum α-foetoprotein is at 16 to 18 weeks of pregnancy (9). In 5 of 120 pregnant women (4.1%) at 16 to 18 weeks of pregnancy, the serum α-foetoprotein was high at 2.5 times the normal median.

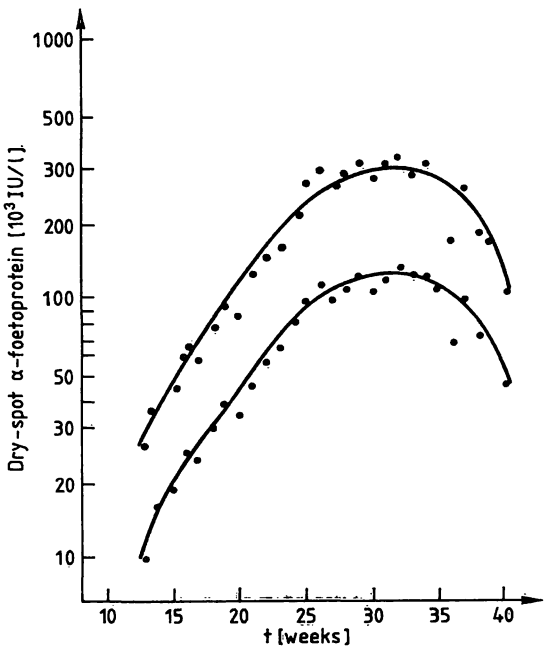


Fig. 5. Dry blood spot α-foetoprotein levels as a function of gestation weeks. Solid lines indicate the median and 2.5 × median of the values obtained.

## Discussion

Currently, RIA or alternative techniques on serum samples are used in screening for neural tube defects by quantifying maternal serum α-foetoprotein. Moreover, dry blood spots have been used successfully in numerous analyses, e. g. for detecting inborn errors of metabolism, congenital hypothyroidism, monitoring concentrations of glucose, cholesterol and therapeutic drugs (22, 23). The essential drawback for α-foetoprotein measurement on dry blood spots has been the need of a reliable assay to ensure an effective and accurate neural tube defect screening. Thus, although the intitial reports showed a good correlation between serum and dry blood tests, they also showed poorer precision and recovery results in the second kind of sample (17, 18, 19). However, mailing blood samples dried on filter paper is more convenient and easier than sending tubes of serum and is the most effective laboratory method for a regional screening programme. Therefore, our objective was to develop a method for measuring maternal serum on dry blood spots, improving the recovery and precision results previously reported.

*Mizejewski* et al. (17) and *Wong* et al. (18) have described the α-foetoprotein stability in dry blood spot samples during mailing and exposure to 4 °C. In our study we have shown that the α-foetoprotein is stable even at higher temperatures (25 and 37 °C) and we have confirmed α-foetoprotein stability in dry blood spots by mailing in a region with mean temperatures higher than those of the reported countries (17, 18). Moreover, we have found that increasing the haematocrit does not affect the results for α-foetoprotein, as also observed in a reported assay of theophylline (22). In the assay, the sensitivity was not better than reported previously for dry blood spots (19), but we achieved better recovery than previously reported (18), and we obtained coefficients of variation for 40 and 160 international kilo-units/l of less than 10%. Besides, the α-foetoprotein results for dry blood spots correlated well with those obtained for serum samples, with no markedly discrepant results even at high α-foetoprotein concentrations.

We think that this method could support the basis of a screening programme for identifying pregnant women with a high risk of neural tube defect. Reinforcing this hypothesis, we found 4.1% of pregnant women with an intial maternal serum α-foetoprotein of 2.5× median. This does not disagree with previously reported results (24—27).

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